

# Evidence for pollinator sharing in Mediterranean nectar-mimic orchids: absence of premating barriers?

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Pollinator specificity has traditionally been considered the main reproductive isolation mechanism in orchids. Among Mediterranean orchids, however, many species attract and deceive pollinators by mimicking nectar-rewarding plants. To test the extent to which deceptive orchid species share pollinators, we collected and identified hemipollinaria-carrying insects, and used ribosomal sequences to identify the orchid species from which hemipollinaria were removed. We found that social and solitary bees, and also flies, carried hemipollinaria belonging to nine orchid species with different degrees of specialization. In particular, *Anacamptis morio*, *Dactylorhiza romana* and *Orchis mascula* used a large set of pollinator species, whereas others such as *Orchis quadripunctata* seemed to be pollinated by one pollinator species only. Out of the insects with hemipollinaria, 19% were found to carry hemipollinaria from more than one orchid species, indicating that sympatric food-deceptive orchids can share pollinators. This sharing was apparent even among orchid sister-species, thus revealing an effective overlap in pollinator sets among closely related species. These results suggest varying degrees of pollinator specificity in these orchids, and indicate that pollinator specificity cannot always act as the main isolation mechanism in food-deceptive temperate orchids.

**Keywords:** bees; hemipollinaria; nuclear rDNA internal transcribed spacer; pollination; orchids; reproductive isolation

## 1. INTRODUCTION

The discoveries by Darwin and numerous other evolutionary biologists and botanists that orchid–pollinator interactions are often highly specific and that a large diversity of orchid–pollination systems exists have led to the hypothesis that orchid species diversity is a consequence of species-specific orchid–pollinator interactions and that genetic barriers are lacking among species (Darwin 1862; Van der Pijl & Dodson 1966; Grant 1994). As a consequence, pollinator specificity has been considered as the main ethological, prezygotic reproductive isolation mechanism among orchid species that grow sympatrically and have overlapping flowering phenologies (Van der Pijl & Dodson 1966; Dressler 1981). The ongoing observations that hybrids are relatively rare between sympatric orchid species has given further support for the important role of pollinator specificity in maintaining orchid species boundaries (Darwin 1862; Van der Pijl & Dodson 1966). This view led researchers to emphasize the importance of prezygotic reproductive isolation mechanisms in orchids. For example, Gill (1989, p. 466) stated that ‘...the integrity of orchid species is maintained by the specializations of the pollinator attraction and visitation rather than by intrinsic genetic barriers’.

A large variety of pollination systems exist in Mediterranean orchids. Plants of the species-rich genus *Ophrys*, for example, are famous for their highly specific pollination by sexual deceit, in which males of one species of solitary bees or wasps pollinate the flowers of one orchid species that mimics the pollinator females through floral scent and shape. In *Ophrys*, reproductive isolation is typically associated with the specificity of pollinator visits (Kullenberg 1961; Schiestl *et al.* 1999; Soliva & Widmer 2003). However, in other Mediterranean orchids, pollination is not always species-specific. An exhaustive collection of pollinator information for European orchids has recently been compiled by Van der Cingel (1995), making the European members of the Orchidinae one of the most extensively studied groups. An analysis of orchid–pollinator relationships reported therein reveals that species-specific relationships evolved in only a few groups, such as *Ophrys*, and that many orchid species seem to be more opportunistic with respect to the choice of their pollinators. Most prominent is a guild of food-deceptive orchids in the genera *Orchis*, *Dactylorhiza*, *Anacamptis* and *Neotinea*, which share large conspicuous flowers, offer no nectar reward to pollinators and are thought to attract and deceive mostly naive pollinators (Dafni 1984, 1987; Nilsson 1992). It is not clear whether food-deceptive species such as these tend towards generalist pollinator attraction in comparison with the sexually deceptive

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orchids that are considered to be specialist. Several studies have been carried out on pollination of food-deceptive Mediterranean orchids, and inspection of available pollinator lists for different food-deceptive orchids (see Van der Cingel 1995 and references therein) indicates that overlap in pollinator species commonly occurs among different orchid species. Unfortunately, previous pollination studies have tended to focus on single orchid species without taking into consideration co-flowering orchid taxa. In particular, information on the potential for pollen movement among different sympatric orchid species via pollinator overlap is entirely missing (but see Nilsson 1984; Aceto *et al.* 1999b).

The observation that hybrids among non-rewarding Mediterranean orchids are relatively rare (Dafni 1987; Pellegrino *et al.* 2000) has promoted the idea that even if different orchid species share pollinator suites, there may be little or no effective pollinator overlap when in sympatry. Therefore, it could be hypothesized that in some areas, depending on the pollinator fauna and the composition of nectar-rewarding model species, some insect species may be preferentially attracted to one orchid species over others. However, when a pollinator of one species by chance visits another sympatric food-deceptive orchid species, hybridization occurs (Dafni 1987).

If pollinator sharing does occur, then it is of interest to know whether it occurs exclusively among distantly related orchid species, or also among very closely related taxa. The latter would imply that these temperate orchid species have evolved alternative reproductive isolation mechanisms for maintaining species boundaries. Today, a well-supported phylogenetic framework for the European Orchidinae is available (Cozzolino *et al.* 2001; Bateman *et al.* 2003), which can be used for investigating pollinator specificity at varying levels of phylogenetic relatedness.

Direct observation of orchid pollination events are notoriously difficult to record, especially in orchids with a deceptive pollination system, since pollination rates are typically low (Neiland & Wilcock 1995). However, this is the crucial information needed for our understanding of the specificity of orchid–pollinator interactions.

To address this problem, we caught insects carrying pollinia in the field and identified the corresponding orchid species by molecular techniques (Widmer *et al.* 2000). In particular, we focused our sampling on meadows where different food-deceptive orchids grow in sympatry and have overlapping flowering phenologies, with the aim of determining whether species effectively share pollinator species and individuals. Specifically, the following questions are addressed.

- (i) Do different orchid species share pollinator species or even individuals?
- (ii) Do certain pollinator taxa express a specific preference for particular orchid species?
- (iii) Are hybrids found in the study area among orchid species that share pollinators?

## 2. METHODS

### (a) *Collecting site*

Insects were collected using hand-nets in contiguous grass meadows in Cilento and Vallo di Diano National Parks (southern Italy) in Spring 2003 and 2004 while they were

foraging on nectar- or pollen-rewarding plants. We selected a study site at 1200 m above sea level that covered an area of 4 km<sup>2</sup> because a large diversity of orchid species has been reported to grow there in sympatry (Nazzaro *et al.* 1995). The vegetation is typical for Apennine limestone grassland. Insects were collected between 25 April and 25 May in 2003 and in 2004 because previous floristic investigations have shown that, during this time, most of the orchid species growing in the area have overlapping flowering periods (Nazzaro *et al.* 1995). During this period, a diversity of nectar-rewarding geophytes are also flowering. In particular, the most common species were *Medicago lupulina* L., *Polygala vulgaris* L., *Primula vulgaris* Hudson, *Thymus longicaulis* C. Presl, *Trifolium pratense* L., *Vicia sativa* L. and *Viola pseudogracilis* Strobl.

The following 21 sympatric species of orchid subtribe Orchidinae were co-flowering in the study area during pollinator collection: *Anacamptis* (*Orchis*) *morio*, *A. (Orchis) papilionacea*, *A. pyramidalis*, *Dactylorhiza romana*, *D. (Coeloglossum) viride*, *Ophrys fuciflora*, *O. lutea*, *O. sphegodes*, *Orchis (Aceras) anthropophora*, *O. mascula*, *O. pauciflora*, *O. provincialis*, *O. purpurea*, *O. quadripunctata*, *O. simia*, *Neotinea maculata*, *N. (Orchis) tridentata*, *N. (Orchis) ustulata*, *Platanthera chlorantha*, *Serapias lingua* and *S. vomeracea*. In addition, the following hybrids were in flower: *O. mascula* × *O. pauciflora*, *A. morio* × *A. papilionacea*, *N. tridentata* × *N. ustulata* and *O. pauciflora* × *O. quadripunctata*. *Himantoglossum (Barlia) robertiana* was also present in the study area but its flowering period preceded the time of our sampling activities, whereas *H. hircinum* flowered only after the sampling period. (For the name authority of all listed orchid species, see Bateman *et al.* 2003).

### (b) *Sample collection and molecular analysis*

Bees and large dipterans foraging on nectar plants in the study area were caught with a butterfly net and those carrying orchid pollen packages were killed, mounted on insect needles, labelled and stored at room temperature in boxes with naphthalene. In contrast to most other flowering plants, orchids typically do not shed individual pollen grains. Instead, pollen grains from one anther are densely packed into a unit that is attached to a sticky plate, the ‘viscidium’. When a pollinator visits an orchid flower, it removes the pollen package because the sticky viscidium becomes attached to a body part of the pollinator. The unit consisting of all pollen from the same anther, plus the viscidium, is called ‘pollinarium’. In some orchids, including the Mediterranean Orchidinae, pollen from one anther is divided into two units, each consisting of a pollen package and a separate viscidium. The two dispersal units are then referred to as ‘hemipollinaria’ (Darwin 1862).

Hemipollinaria and their remains (i.e. the viscidium and the structure carrying the pollen grains that remain after pollen deposition) were removed from insects with forceps and stored in polymerase chain reaction (PCR) vials at 4 °C. For simplicity, we will hereafter refer to all hemipollinaria and their remains simply as ‘hemipollinaria’. Pollinators were identified by comparison with reference specimens from the Zürich Eidgenössische Technische Hochschule (Institute for Special Botany) insect collection. The delimitation of bee genera follows Michener (2000). DNA from single hemipollinaria was extracted and the nuclear ribosomal internal transcribed spacer 1 (ITS1) region was PCR amplified as described in Widmer *et al.* (2000). In cases where only an

Table 1. Number of individuals per insect species carrying orchid hemipollinaria.

	<i>Anacamptis morio</i>	<i>Anacamptis papilionacea</i>	<i>Dactylorhiza romana</i>	<i>Neotinea tridentata</i>	<i>Orchis mascula</i>	<i>Orchis pauciflora</i>	<i>Orchis provincialis</i>	<i>Orchis quadripunctata</i>	<i>Ophrys</i> sp.
<i>Andrena albopunctata</i> (Rossi 1792)	1	—	—	—	—	—	—	—	—
<i>A. ovatula</i> group	1	—	—	—	—	—	—	—	—
<i>Anthophora aestivalis</i> (Panzer 1801)	—	—	1	—	—	—	—	—	—
<i>A. mucida</i> (Gribodo 1873)	—	—	1	—	—	1	—	—	—
<i>A. plumipes</i> (Pallas 1772)	—	—	—	—	—	1	—	—	—
<i>A. retusa</i> (Linnaeus 1758)	—	—	—	—	—	—	—	—	2
<i>Apis mellifera</i> (Linnaeus 1758)	1	—	—	1	—	—	—	—	—
<i>Bombus hortorum</i> (Linnaeus 1761)	—	—	2	—	2	—	—	—	—
<i>B. humilis</i> (Illiger 1806)	10	1	10	—	1	4	1	—	—
<i>B. lapidarius</i> (Linnaeus 1758)	4	—	9	—	4	—	—	—	—
<i>B. pratorum</i> (Linnaeus 1761)	—	—	—	—	1	—	—	—	—
<i>B. ruderarius</i> (Müller 1776)	—	—	—	—	1	—	—	—	—
<i>B. ruderatus</i> (Fabricius 1775)	1	—	2	—	3	—	—	—	—
<i>B. rupestris</i> (Fabricius 1793)	3	—	—	—	1	—	—	—	—
<i>B. sylvarum</i> (Linnaeus 1761)	6	—	2	—	—	—	—	—	—
<i>B. sylvestris</i> (Lepeletier 1832)	1	—	1	—	—	—	—	—	—
<i>B. terrestris</i> group	6	—	5	—	6	—	—	—	—
<i>B. vestalis</i> (Geoffroy 1785)	3	—	—	—	—	—	—	—	—
<i>Bombus major</i> (Linnaeus 1758)	—	—	—	—	1	—	—	2	—
<i>Eucera hungarica</i> (Friese 1895)	1	1	1	—	1	—	2	—	—
<i>E. nigrescens</i> (Pérez 1879)	—	9	1	—	—	—	1	—	—
<i>Halictus patellatus</i> (Morawitz 1873)	1	—	—	1	—	—	—	—	—
<i>H. sexinctus</i> (Fabricius 1775)	—	—	—	—	1	—	—	—	—
<i>Lasioglossum xanthopus</i> (Kirby 1802)	1	—	—	—	—	—	—	—	—
<i>Osmia bicornis</i> (Linnaeus 1758)	1	—	—	1	—	—	—	—	—
<i>O. niveata</i> (Fabricius 1804)	—	—	—	1	—	—	—	—	—
number of insect species	15	3	11	4	11	3	3	1	1

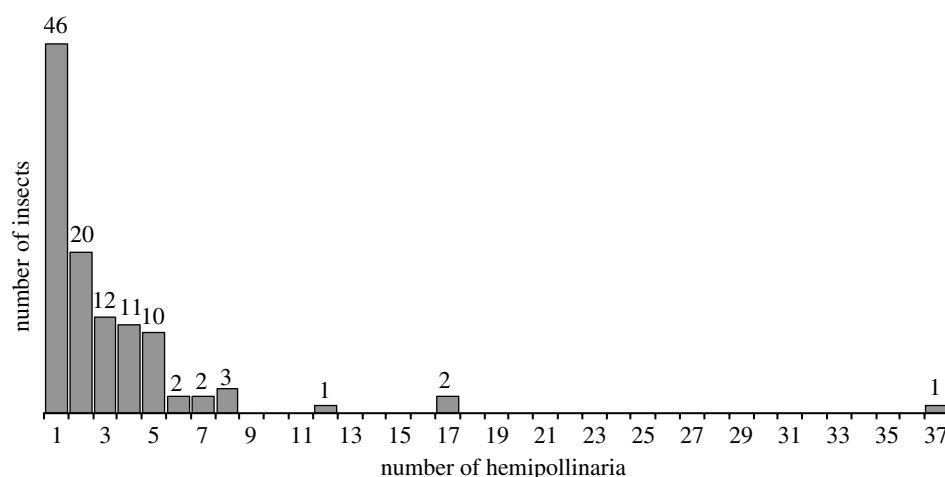


Figure 1. Number of pollinators carrying different numbers of hemipollinaria. Most individuals were found to carry only a single hemipollinarium; the maximum number of hemipollinaria found on a single insect was 37.

extremely small amount of material was available, ITS1 was amplified directly without prior isolation of DNA by adding the biological material directly to the PCR reaction mixture.

PCR products generated with ITS primers were sequenced according to Aceto *et al.* (1999a). To identify the origin of pollinaria, ITS sequences were subjected to a FASTA search against GenBank or compared with unpublished orchid sequences datasets (Cozzolino *et al.* 2001; Bateman *et al.* 2003). In one case (*D. romana*), confirmatory sequences were obtained from plant material collected at the study site, as a single mismatch was observed between the pollinarium sample and the published sequence.

### 3. RESULTS

A total of 1375 bees and large dipterans were captured during the study period and checked for the presence of hemipollinaria. Out of these, 109 individuals (8%) carried at least one hemipollinarium and were collected (32 in spring 2003, 77 in spring 2004; for insect list, see Electronic Appendix, part A). All other insects were released immediately. A total of 347 hemipollinaria was recovered and analysed from these 109 bees and large dipterans. PCR products were successfully obtained from 321 (92.5%) of the hemipollinaria (table 1), and we were able to identify hemipollinaria from 105 out of the 109 collected pollinators. Out of the 109 bees and large dipterans, 46 carried a single hemipollinarium, of which we were able to identify 43. Sixty-three pollinators carried more than one hemipollinarium, of which we identified hemipollinaria from 62 of them (figure 1); the mean number of hemipollinaria per bee was  $2.33 (\pm 2.62)$  and the median was 2. Among the 62 insects with more than one hemipollinarium, 42 carried hemipollinaria of a single orchid species, 18 had hemipollinaria from two different orchid species, and two insects bore hemipollinaria from three different orchid species (table 2, figure 2). Overall, 85 pollinators were found with one or more hemipollinaria from a single orchid species, whereas 20 carried hemipollinaria from different orchid species (figure 2).

Hemipollinaria from 9 out of the 21 orchid species present in the study area were found in our molecular analyses. Although hemipollinaria of some orchid species were found on a range of pollinator species, others were

present only on a single pollinator. In particular, hemipollinaria of *O. mascula* and of *D. romana* were found on 11 different pollinator species and those of *A. morio* were found on 15 different pollinator species. By contrast, hemipollinaria of *A. papilionacea* were mostly found on males of two *Eucera* species, and hemipollinaria of *O. quadripunctata* were found exclusively on *Bombylius* flies (table 1). We did not find evidence that hemipollinaria from different orchid species were placed on different parts or in different positions on the pollinators' heads (see Electronic Appendix, part B).

The number of identified hemipollinaria varied significantly among orchid species (table 2), with those of *D. romana*, *O. mascula* and *A. morio* being the most common (80% of the identified hemipollinaria belonged to these three species). Accordingly, the largest number of insects were found to be pollinators of these three species: *D. romana* (35 insects), *O. mascula* (22 insects) and *A. morio* (41 insects).

Grouping insects according to ecological behaviour indicated that bumble-bees (species of the genus *Bombus*) were the most common pollinators. But a significant contribution to orchid pollination was also offered by solitary bees (figure 3). Significantly, one single honeybee (*Apis mellifera*) was found carrying 37 hemipollinaria, but only from two orchid species.

To estimate levels of pollinator specificity, we considered the number of insects that carried hemipollinaria exclusively from one orchid species (specific pollinators), and the number of insects that carried hemipollinaria from two or more orchid species (non-specific pollinators). For the analysis, we removed all insects that carried a single hemipollinarium (46 insects) because a single visit (when one hemipollinarium can be removed) provided no information on the specificity of pollinator preferences. At the same time, we were not able to discriminate whether insects carrying two hemipollinaria of the same orchid species picked them up from the same flower (one visit) or from two different flowers (two visits), because during a single visit, one or two hemipollinaria can be randomly removed. However, we used a conservative approach in our estimation of specific pollinators by assuming that insects that carried two hemipollinaria picked them from two visits (i.e. we included them among the specific pollinators; table 3).



Table 2. Number of hemipollinaria and number of insects carrying one or more hemipollinaria of each orchid species.

	<i>A. morio</i>	<i>A. papilionacea</i>	<i>D. romana</i>	<i>N. tridentata</i>	<i>O. mascula</i>	<i>O. pauciflora</i>	<i>O. provincialis</i>	<i>O. quadripunctata</i>	<i>Ophrys</i> sp.
number of hemipollinaria found (% of total)	98 (31)	22 (7)	89 (28)	17 (5)	65 (21)	8 (3)	8 (3)	2 (1)	6 (2)
number of insects carrying hemipollinaria (% of total)	41 (32)	11 (9)	35 (28)	4 (3)	22 (17)	6 (5)	4 (3)	2 (2)	2 (2)
insects with one hemipollinarium	17	3	10	2	5	2	2	2	0
insects with at least two hemipollinaria from the orchid	24	8	25	2	17	4	2	0	2

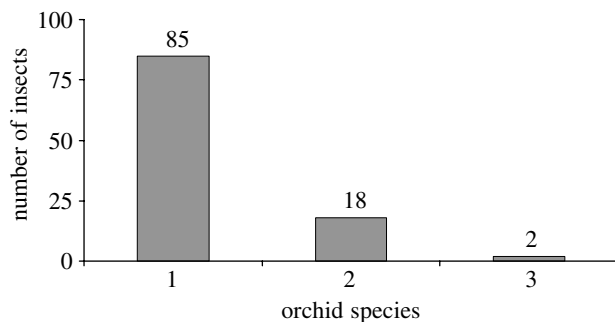


Figure 2. Number of insects carrying hemipollinaria derived from one, two or three orchid species.

To test for significant difference between the frequency of pollinators with more than one conspecific hemipollinarium (specific pollinators) and more than one hetero-specific hemipollinarium (non-specific pollinators), we performed a  $\chi^2$  test (expected frequencies: 50 : 50) by using the SPSS statistic software package for the three orchid species (namely, *A. morio*, *D. romana* and *O. mascula*), from which we gathered a relevant number of pollination events (figure 4). We did not find significant differences between specific and non-specific pollinators among the three species (*A. morio*:  $\chi^2_1 = 0$ ,  $p = 1$ ; *D. romana*:  $\chi^2_1 = 1.815$ ,  $p = 0.168$ ; *O. mascula*:  $\chi^2_1 = 0.529$ ,  $p = 0.467$ ).

Interestingly, six ITS sequences, which did not match with any orchid sequence, identified nectar- and pollen-rewarding non-orchid species present in the investigated area. These sequences originated from pollen grains attached to the hemipollinaria because of previous visits of the pollinator to nectar- or pollen-offering flowers.

#### 4. DISCUSSION

Although low fruit set in many plants is attributed to resource limitation for fruit development, in non-rewarding orchids, evidence suggests that this phenomenon is due to pollination limitation (Neiland & Wilcock 1995). Accordingly, in our survey, only a small proportion of collected insects was found carrying orchid pollinaria (ca. 8%). All insects that carried hemipollinaria had them attached to the head, as has been previously observed (Nilsson 1980). The insects carried pollinaria of nine of the 21 orchid species present in the area. We failed to retrieve hemipollinaria of *A. pyramidalis*, *D. viride*, *O. anthropophora*, *O. purpurea*, *O. simia*, *N. maculata*,

*N. ustulata*, *P. chlorantha*, *S. lingua* and *S. vomeracea*, although some of these orchid species were quite common in the study area. For *N. maculata*, this result is not surprising given that the species is predominantly auto-gamous (Van der Cingel 1995). The limited available pollinator information for the other species (see Van der Cingel 1995 and references therein) indicates that they are pollinated primarily by moths (*A. pyramidalis*, *P. chlorantha*), small coleopterans (*O. anthropophora*, *O. simia*) and small wasps (*N. ustulata*, *C. viridae*). The absence of pollinaria from these orchid species in our samples is thus most probably a consequence of our sampling strategy, which was focused on bees and large dipterans. A different insect-sampling strategy (i.e. insect traps) would be required to determine the pollinators of these orchid species.

Although, in some circumstances, it may be possible to recognize the origin of hemipollinaria by morphological difference, only molecular analyses allowed reliable identification because morphological differences between hemipollinaria, particularly from closely related orchid species, are often lacking (Barone Lumaga *et al.* 2000).

Hemipollinaria of *D. romana*, *O. mascula* and *A. morio* were found on different bee species, thus confirming previous findings that show these species are pollinated by different pollinators (Nilsson 1980, 1983, 1984). The three orchid species, and the others from which we recovered hemipollinaria in this study, are characterized by large and conspicuous flowers provided with a spur. By contrast, hemipollinaria were absent from those orchid species with different floral morphology (i.e. *C. viridae* and *N. ustulata*, which have small labella, and *O. anthropophora*, which lacks a spur). Our findings support the hypothesis of Sabat & Ackerman (1996) that there is a trend towards a common floral morphology in non-rewarding orchid species to promote the preferential attraction of solitary bees and bumble-bees as pollinators.

The most common orchid species in our dataset, *O. mascula*, *A. morio* and *D. romana*, have been extensively investigated in the northern part of their distribution by Nilsson (1980, 1983, 1984). For these three species, Nilsson reported that bumble-bee queens were the main pollinators. In particular, bumble-bees were almost the exclusive pollinators (up to 92%) in northern populations of *A. morio*. By contrast, we found that in the Mediterranean region, not only bumble-bee queens but also the solitary bees from the genera *Andrena*, *Halictus* and *Eucera*

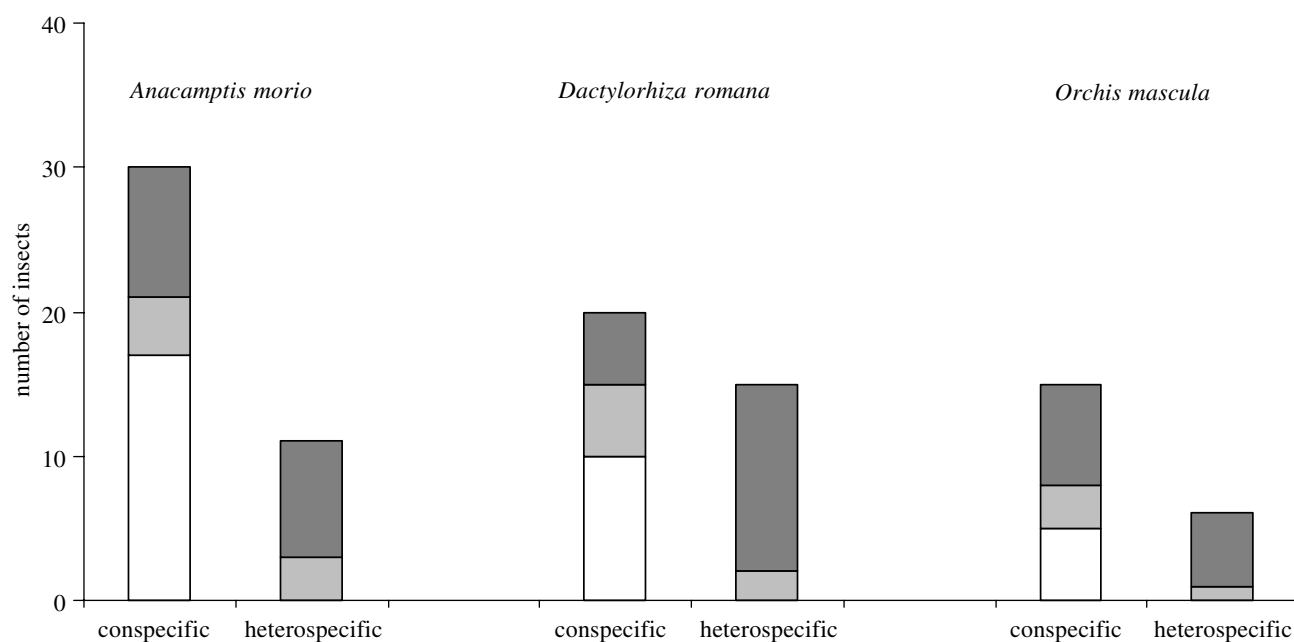


Figure 3. Specific and non-specific pollinators of *A. morio*, *D. romana* and *O. mascula*. The first bar shows the number of insects carrying only conspecific hemipollinaria. The second bar shows the number of insects carrying heterospecific hemipollinaria (from more than one orchid species). White, one hemipollinarium; light grey, two hemipollinaria; dark grey, more than two hemipollinaria.

play a prominent pollinator role in *A. morio*. This discordance between the two regions indicates the importance of making pollinator observations across the entire species range. The European Orchidinae have diversified in Mediterranean regions and it is here that we are most likely to observe those pollinators that have been actively involved in the evolution of these deceptive orchids (Nilsson 1984).

Out of the pollinators, 58% were found carrying more than one hemipollinarium. NrITS1 sequence analysis revealed that the same insect species can act as a pollinator for different sympatric, co-flowering orchid species, and that often hemipollinaria found on the same individual pollinator originate from different orchid species. This result demonstrates an effective sharing of pollinators among nectar-mimicking sympatric orchid species. This sharing of individual pollinators is not restricted to distantly related orchid species. For example, hemipollinaria of the closely related species *O. mascula* and *O. pauciflora*, as indicated by phylogenetic studies (Cozzolino *et al.* 2001; Bateman *et al.* 2003), were found on the same individual insect (*Bombus humilis*), confirming the occurrence of pollinator sharing also among orchid sister-species.

Out of the 109 insects with hemipollinaria, 63 carried two or more hemipollinaria. In 20 of these cases, the pollinaria originated from at least two different orchid species. Consequently, about one-third of all pollinators that had visited more than a single orchid flower carried hemipollinaria from different species. This estimate of pollinator sharing is conservative, as two hemipollinaria of the same orchid species on a given pollinator could have been picked up by a single flower visit (see above).

Pollinator sharing differed among sympatric orchid species, perhaps because of different pollinator-attraction strategies. For *O. mascula*, for example, we found hemipollinaria on several *Bombus*, *Eucera* and *Bombylius* species (see table 1). By contrast, hemipollinaria of

*A. papilionacea* were found almost exclusively on *Eucera* males, and *O. quadripunctata* hemipollinaria were found for the first time on the long-tongued *Bombylius* flies only. While the association of *A. papilionacea* with *Eucera* males has previously been proposed by Vogel (1972) based on insect behaviour observations, the pollination of *O. quadripunctata* by *Bombylius* has not been reported to date.

Interestingly, these observations illustrate that pollinator specificity does not necessarily rescue orchid species from hybridization with and introgression from closely related species. The *Eucera* males, which were found to be the main pollinators of *A. papilionacea*, also carried hemipollinaria of *A. morio*, the sister species of *A. papilionacea*. Similarly, on *Bombylius*, we found not only hemipollinaria of the pollinator-specific *O. quadripunctata*, but also of the closely related *O. mascula*. Evidence that such unilateral pollinator promiscuity has evolutionary consequences is provided by the presence of interspecific hybrids between *O. mascula* and *O. quadripunctata* on the one hand, and between *A. morio* and *A. papilionacea*, on the other.

Although hybridization and introgression occur, it is surprisingly rare among Mediterranean orchids (Aceto *et al.* 1999b; Pellegrino *et al.* 2000) given the frequency with which individual pollinators carry hemipollinaria from more than one orchid species. Out of many possible hybrid combinations resulting from co-occurrence of hemipollinaria on the same insect, only four hybrid taxa have been found in the field (two of them, namely *O. mascula* × *O. pauciflora* and *A. morio* × *A. papilionacea*, are predicted by the pollinator sharing evidence). Mechanisms other than pollinator specificity must therefore act to maintain species boundaries among these temperate food-deceptive orchids. Different post-mating mechanisms involving pollen–pistil interactions, hybrid embryo development or later post-zygotic barriers may halt interspecific gene flow. Recent evidence shows that

Table 3. Sharing of pollinators among orchid species.

(Number of insects carrying more than one hemipollinarium of one or two orchid species. The number of insects carrying more than two hemipollinaria of the same orchid species is given in brackets.)

	<i>A. morio</i>	<i>A. papilionacea</i>	<i>D. romana</i>	<i>N. tridentata</i>	<i>O. mascula</i>	<i>O. pauciflora</i>	<i>O. provincialis</i>	<i>O. quadripunctata</i>	<i>Ophrys</i> sp.
<i>A. morio</i>	13 (9)	—	8	2	2	—	1	—	—
<i>A. papilionacea</i>	—	5 (2)	2	—	—	—	1	—	—
<i>D. romana</i>	8	2	10 (5)	—	4	2	1	—	—
<i>N. tridentata</i>	2	—	—	—	—	—	—	—	—
<i>O. mascula</i>	2	—	4	—	10 (7)	1	—	—	—
<i>O. pauciflora</i>	—	—	2	—	1	1	—	—	—
<i>O. provincialis</i>	1	1	1	—	—	—	—	—	—
<i>O. quadripunctata</i>	—	—	—	—	—	—	—	—	—
<i>Ophrys</i> sp.	—	—	—	—	—	—	—	—	2 (2)
specific/unspecific pollinators	13/13	5/3	10/17	0/2	10/7	1/3	0/3	0/0	2/0

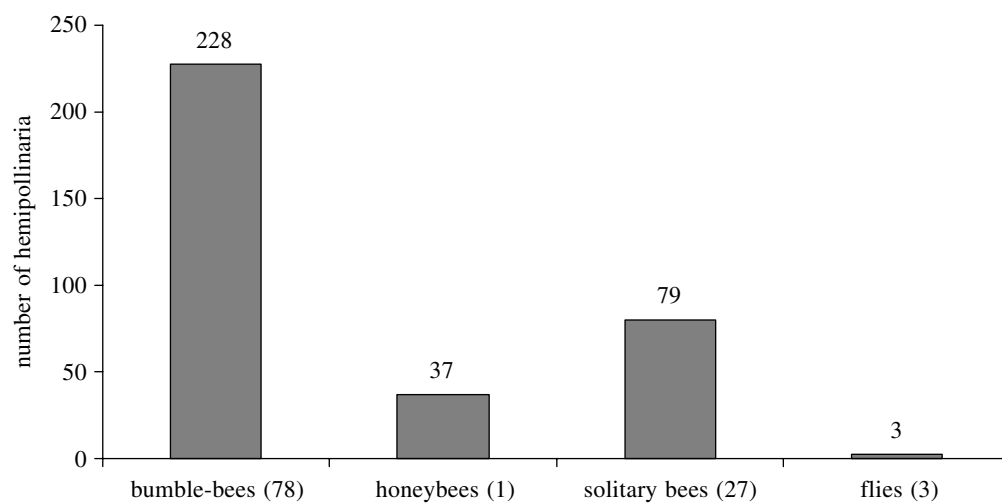


Figure 4. Number of individual insects carrying hemipollinaria according to insect group. The numbers of individuals are given in brackets. Bumble-bees (including queens, workers and males) were the most frequent pollinators, but solitary bees contributed widely to pollination.

orchid species-pairs with a generalized pool of pollinators have significantly more divergent karyotypes than species-pairs with different pollinators (Cozzolino *et al.* 2004). These results suggest that karyotype differences acting as late postzygotic reproductive barriers may play an important role in the maintenance of species boundaries in food-deceptive orchids that share pollinators.

Sharing of pollinators among closely related orchid species represents the most striking difference from other orchid groups in which strong pollinator specificity occurs, such as in many tropical orchids and in the temperate sexually deceptive *Ophrys* species (Van der Pijl & Dodson 1966; Schiestl *et al.* 1999; Soliva & Widmer 2003). However, our results highlight the absence of significant premating isolation mechanisms that act to maintain species boundaries among food-deceptive orchids.

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## REFERENCES

- Aceto, S., Caputo, P., Cozzolino, S., Gaudio, L. & Moretti, A. 1999a Phylogeny and evolution of *Orchis* and allied genera based on ITS DNA variation: morphological gaps and molecular continuity. *Mol. Phylogenet. Evol.* **13**, 67–76.
- Aceto, S., Cozzolino, S., Gaudio, L., Nazzaro, R. & De Luca, P. 1999b Pollination flow in hybrid formation between *Orchis morio* and *O. papilionacea* (Orchidaceae) in two different habitats. *Int. J. Plant Sci.* **160**, 1153–1156.
- Barone Lumaga, M. R., Caputo, P., Cozzolino, S. & Tarallo, R. 2000 Pollinaria and massulae surface morphology in the subtribe Orchidinae. In *Proceedings of Orchis 2000 Conference, San Zeno di Montagna, Italy*, pp. 5–6.
- Bateman, R. M., Hollingsworth, P. M., Preston, J., Yi-Bo, L., Pridgeon, A. M. & Chase, M. W. 2003 Molecular phylogenetics and evolution of Orchidinae and selected Habenariinae (Orchidaceae). *Bot. J. Linn. Soc.* **142**, 1–40.
- Cozzolino, S., Aceto, S., Caputo, P., Widmer, A. & Dafni, A. 2001 Speciation processes in Eastern Mediterranean *Orchis* s.l. species: molecular evidence and the role of pollination biology. *Isr. J. Plant Sci.* **49**, 91–103.

- Cozzolino, S., D'Emérico, S. & Widmer, A. 2004 Evidence for reproductive isolate selection in Mediterranean orchids: karyotype differences compensate for the lack of pollinator specificity. *Proc. R. Soc. B* **271**, 259–262. (doi:10.1098/rspb.2004.0166.)
- Dafni, A. 1984 Mimicry and deception in pollination. *Annu. Rev. Ecol. Syst.* **15**, 259–278.
- Dafni, A. 1987 Pollination in *Orchis* and related genera: evolution from reward to deception. In *Orchid biology: reviews and perspectives* (ed. J. Arditti), pp. 79–104. Ithaca: Cornell University Press.
- Darwin, C. 1862 *The various contrivances by which British and foreign orchids are fertilized by insects*. London: Murray.
- Dressler, R. L. 1981 *The orchids: natural history and classification*. Cambridge, MA: Harvard University Press.
- Gill, D. E. 1989 Fruiting failure, pollination inefficiency, and speciation in orchids. In *Speciation and its consequences* (ed. D. Otte & J. A. Endler), pp. 458–481. Philadelphia: Academy of Natural Sciences Publications.
- Grant, V. 1994 Modes and origins of mechanical and ethological isolation in angiosperms. *Proc. Natl Acad. Sci. USA* **91**, 3–10.
- Kullenberg, B. 1961 Studies in *Ophrys* pollination. *Zool. Bidrag. Uppsala* **34**, 1–340.
- Michener, C. D. 2000 *The bees of the world*. Baltimore: The Johns Hopkins University Press.
- Nazzaro, R., Menale, B. & Di Novella, N. 1995 Le Orchidaceae della zona occidentale del Vallo di Diano (Salerno). *Webbia* **50**, 25–35.
- Neiland, M. R. M. & Wilcock, C. C. 1995 Maximisation of reproductive success by European Orchidaceae under conditions of infrequent pollination. *Protoplasma* **187**, 39–48.
- Nilsson, L. A. 1980 The pollination ecology of *Dactylorhiza sambucina* (Orchidaceae). *Bot. Not.* **133**, 367–385.
- Nilsson, L. A. 1983 Anthecology of *Orchis mascula* (Orchidaceae). *Nord. J. Bot.* **3**, 157–179.
- Nilsson, L. A. 1984 Anthecology of *Orchis morio* (Orchidaceae) at its outpost in the north. *Nova Acta Reg. Soc. Sci. Upsal.* **5**, 167–179.
- Nilsson, L. A. 1992 Orchid pollination biology. *Trends Ecol. Evol.* **7**, 255–259.
- Pellegrino, G., Caputo, P., Cozzolino, S., Menale, B. & Musacchio, A. 2000 Molecular characterization of a hybrid zone between *O. mascula* (L.) L. and *O. pauciflora* Ten. (Orchidaceae) in Southern Italy. *Biol. Plant.* **43**, 13–18.
- Sabat, A. M. & Ackerman, J. D. 1996 Fruit set in a deceptive orchid: the effect of flowering phenology, display size, and local floral abundance. *Am. J. Bot.* **83**, 1181–1186.
- Schiestl, F. P., Ayasse, M., Paulus, H. F. L., Löfstedt, C., Hansson, B. S., Ibarra, F. & Francke, W. 1999 Orchid pollination by sexual swindle. *Nature* **399**, 421–422.
- Soliva, M. & Widmer, A. 2003 Gene flow across species boundaries in sympatric, sexually deceptive *Ophrys* (Orchidaceae) species. *Evolution* **57**, 2252–2261.
- Van der Cingel, N. A. 1995 *An atlas of orchid pollination—European orchid*. Rotterdam: Balkema.
- Van der Pijl, L. & Dodson, C. H. 1966 *Orchid flower: their pollination and evolution*. Coral Gables: University Miami Press.
- Vogel, S. 1972 Pollination von *Orchis papilionacea* L. in den Schwarmbahnen von *Eucera tuberculata* F. *Jahresber. Naturwiss. Ver. Wuppertal* **25**, 67–74.
- Widmer, A., Cozzolino, S., Pellegrino, G., Soliva, M. & Dafni, A. 2000 Molecular analysis of orchid pollinaria and pollinaria-remains found on insects. *Mol. Ecol.* **9**, 1911–1914.

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